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PRINCIPAL INVESTIGATOR: Donato F. Romagnolo

CONTRACTING ORGANIZATION: The University of Arizona
Tucson, AZ 85719

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14. ABSTRACT

Information from animal models and population studies suggest that mammary tumor promotion in adult life is influenced by prior exposure to carcinogens in early life. The main **purpose** of this project is to investigate whether or not activation of the aromatic hydrocarbon receptor (AhR) induces CpG methylation at the BRCA-1 gene, and if this epigenetic event predisposes to development of triple-negative breast cancers (TNBC). **Major findings:** Preliminary data acquired through the support of this grant indicate that: 1) targeting of the AhR with an AhR antagonist in cell culture experiments with human breast cancer cells harboring hypermethylated BRCA-1 reactivates BRCA-1 and estrogen receptor- α expression; 2) Comparative analyses of human breast tumors indicate the existence of a correlation between higher BRCA-1 promoter methylation and overexpression of AhR in TNBC, but not in luminal type A and B, or Her2-positive breast cancers; and 3) We are developing colonies through breeding of AhR and BRCA-1 conditional mammary tissue knockouts that will allow us to test the interaction between BRCA-1 and AhR genotype on developmental effects of AhR activation on BRCA-1 expression and promoter methylation. **Significance:** these data provide preliminary evidence that overexpression/activation of the AhR contributes to a TBNC phenotype characterized by increased BRCA-1 promoter methylation.

15. SUBJECT TERMS

BRCA-1, aromatic hydrocarbon receptor, BRCA-1 promoter methylation, triple-negative breast cancers, estrogen receptor-alpha

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1. INTRODUCTION

Subject: The BRCA-1 gene encodes a tumor suppressor protein involved in DNA repair and transcription control (1-5). **Purpose:** sporadic breast cancers, which represent the vast majority (~90%) of breast tumor cases, do not have mutations in the BRCA-1 gene (BRCA-1^{+/+}), but have absent or markedly reduced levels of BRCA-1 similar to those observed in hereditary BRCA-1 tumors (6-12). **Scope:** understanding the mechanisms that contribute to silencing of BRCA-1 has important implications for the prevention of both hereditary and sporadic breast cancers.

2. KEYWORDS

BRCA-1, triple-negative breast cancer, AhR, epigenetic, estrogen receptor- α , hereditary breast cancer, sporadic breast cancer.

3. ACCOMPLISHMENTS

○ What were the major goals of the project?

Goal 1. Investigate interactions between activation of the AhR and BRCA-1 genotype and impact on CpG promoter methylation associated with the TNBC phenotype;

Goal 2. Investigate the combinatorial effects of gestational and postpubertal activation of the AhR on CpG promoter methylation of BRCA-1 and the development of TNBC mammary tumors.

○ What was accomplished under these goals?

❖ *Accomplishment 1*

One of the main questions raised by this project is whether or not silencing of BRCA-1 is linked to overexpression/activation of the AhR. To begin answering this question and as a proof-of-principle for confirmation/control of BRCA-1 and AhR laboratory assays, we have gathered preliminary data from human breast tumors collected at The University of Arizona Cancer Center. We performed analyses of RNA expression for the AhR and in parallel collected genomic DNA from 20 tumors. The tumors were organized based on pathological examinations in 5 subgroups:

- 1) Basal-like TNBC, ER-negative, PR-negative, Her-2 negative – cytokeratin-negative, and epidermal growth factor receptor-negative;
- 2) Her-2-positive, ER-negative;
- 3) Luminal type A (LUM-A), ER-positive and/or PR-positive, Her-2-negative;
- 4) Luminal type B (LUM-B); ER-positive and/or PR-positive, Her-2 positive;
- 5) Non-tumor (NT) controls.

First, we harvested total RNA from the tumor tissue. mRNAs were quantitated via nanodrop measurement and expression levels were measured by quantitative real-time PCR (qRT-PCR). The preliminary data summarized in Figure 1 suggested that average expression of the AhR mRNA was highest in TNBC (~4.2-fold) compared to NT-CONTROL. Conversely, average mRNA levels were reduced in LUM-A (~0.88-fold), Her-2-positive (~0.53-fold), and LUM-B (~0.41-fold) compared to NT-CONTROL. There was large variability in the expression of AhR in TNBC ranging from 2.2-fold to ~8.0-fold. These differences may be due to TBNC heterogeneity as well as stage of tumor development. In contrast the fold-reduction in AhR expression was similar for the other tumor types (0.41-0.88-fold). These data were in agreement with the hypothesis increased expression of AhR may be associated with the development of TNBC.

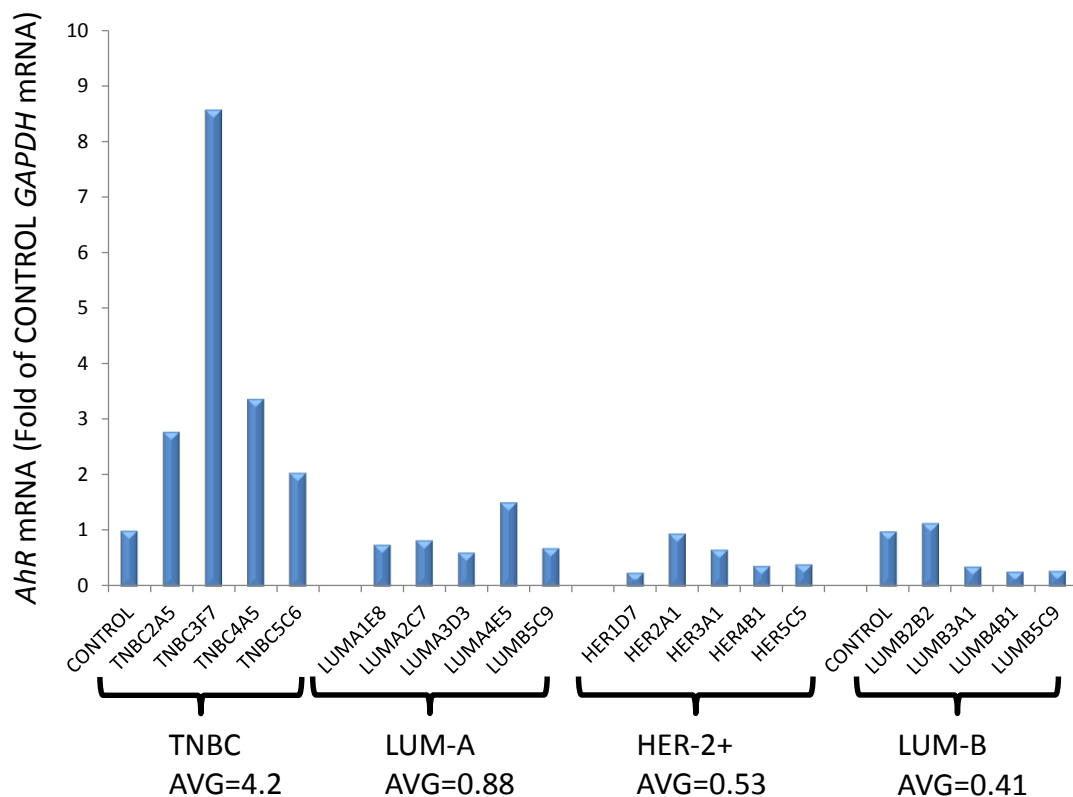


Figure 1. Expression of AhR mRNA in human breast tumors. Bars represent AhR mRNA corrected for GAPDH in control human breast tissue (CONTROL), and TNBC, LUM-A, HER-2+, and LUM-B breast tumors. Numbers below tumor types represent average (AVG) fold-changes in AhR mRNA compared to non-tumor CONTROL. Each bar is the average from at list six replicates/tumor.

Based on these results, we examined changes in BRCA-1 promoter methylation in control and breast tumor tissues. This approach was based on the current hypothesis overexpression of the AhR may be associated with epigenetic repression of BRCA-1 via promoter CpG methylation. Genomic DNA was prepared from breast control tissue and tumors, and quantitated via nanodrop measurement. After bisulfonation of DNA, we performed qRT-PCR BRCA-1 promoter amplification with unmethylated and methylated-specific BRCA-1 primers. BRCA-1 methylation (M) data were normalized against non-methylated BRCA-1 (M/U). Data presented in Figure 2 illustrated that in TNBC BRCA-1 promoter was highly methylated (on average ~5.7-fold) compared to non-tumor CONTROL tissue. Conversely, BRCA-1 promoter methylation was lower in LUM-A (0.48-fold), LUM-B (0.70-fold), and HER-2+ (0.5-fold).

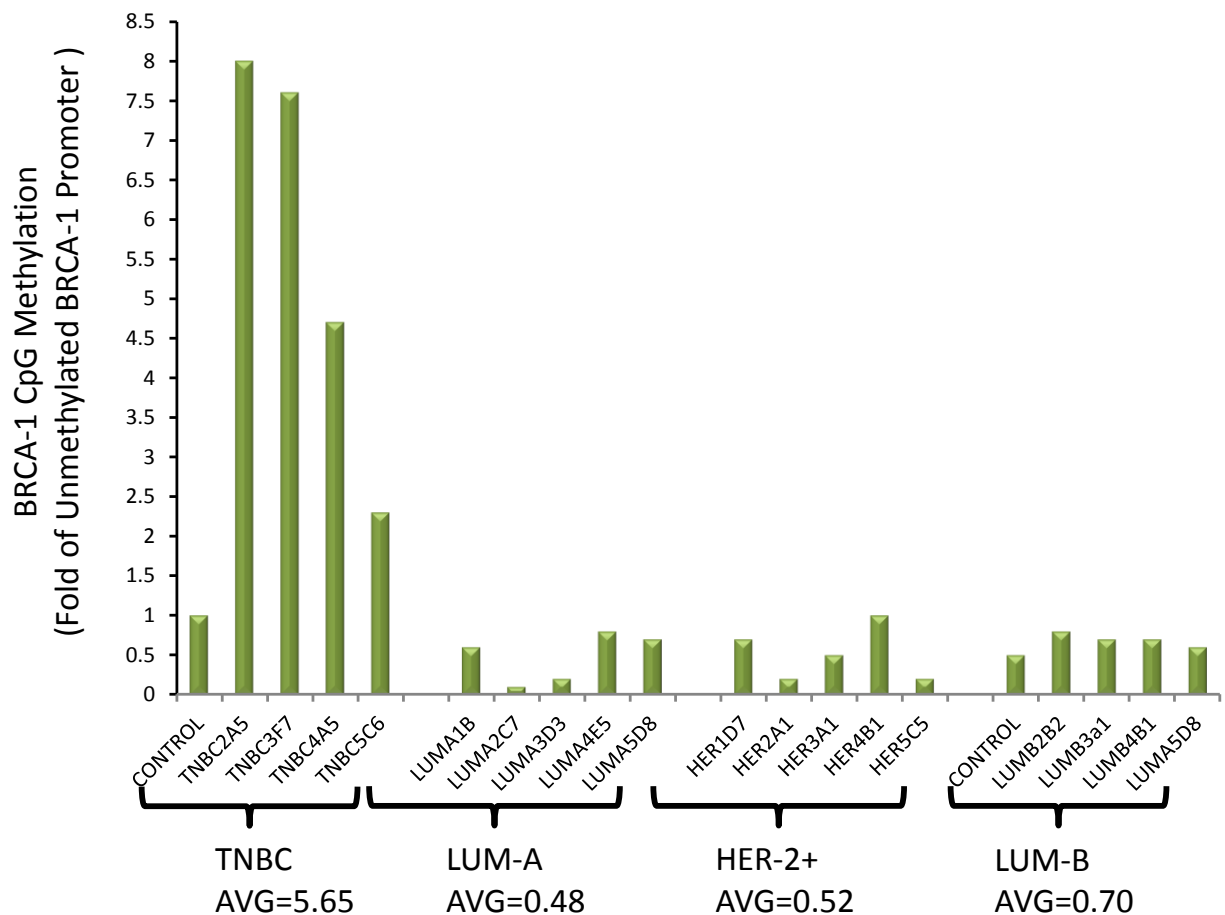


Figure 2. BRCA-1 promoter CpG methylation in human breast tumors. Bars represent CpG methylated BRCA-1 promoter corrected for unmethylated BRCA-1 promoter (M/U) in control human breast tissue (CONTROL), and TNBC, LUM-A, HER-2+, and LUM-B breast tumors. Numbers below tumor types represent average (AVG) fold-changes in BRCA-1 CpG methylation compared to non-tumor CONTROL. Each bar is the average from at least six replicates/tumor.

❖ Accomplishment 2

The expression and methylation data provided in Figure 1 and Figure 2, albeit preliminary, support to the conclusion increased expression of the AhR tends to cluster with the TNBC phenotype. Because the BRCA-1 is a molecular target for the AhR, this provides a new mechanistic link between overexpression of this receptor and development of TNBC. These types of tumors do not have a targeted therapy, and these data may suggest that targeting the AhR either via either inactivation or reduced expression may offer a new molecular target for prevention.

To this end, we first compared the expression of BRCA-1 and AhR in human ER α -positive MCF-7 and ER α -negative UACC-3199 sporadic breast cancer cells. We selected these cell lines based on the knowledge MCF-7 cells express wild-type BRCA-1 and ER α , whereas UACC-3199 cells have wild-type but hypermethylated *BRCA-1* gene(13, 14), and express low levels of ER α (15). Results of Western blot analysis (Figure 3A) revealed that expression of BRCA-1 protein was higher in MCF-7 (~5.0-fold) compared to UACC-3199 cells. Conversely, the expression of the AhR was notably higher (~15.0-fold) in UACC-3199 compared to MCF-7 cells (Figure 3B). As a control, we compared the expression of AhR mRNA and found that it was higher in UACC-3199 cells compared to MCF-7 cells. Further as a positive control for AhR activity, we found that expression of the AhR target gene, CYP1B1 was increased ~7-fold in UACC-3199 cells indicating the AhR pathway is constitutively active in these cells. Because UACC-3199 cells have hypermethylated BRCA-1 promoter and constitutively expressed AhR, we concluded that in fact an inverse relationship may exist between repression of BRCA-1 expression via promoter methylation and overexpression/activation of the AhR

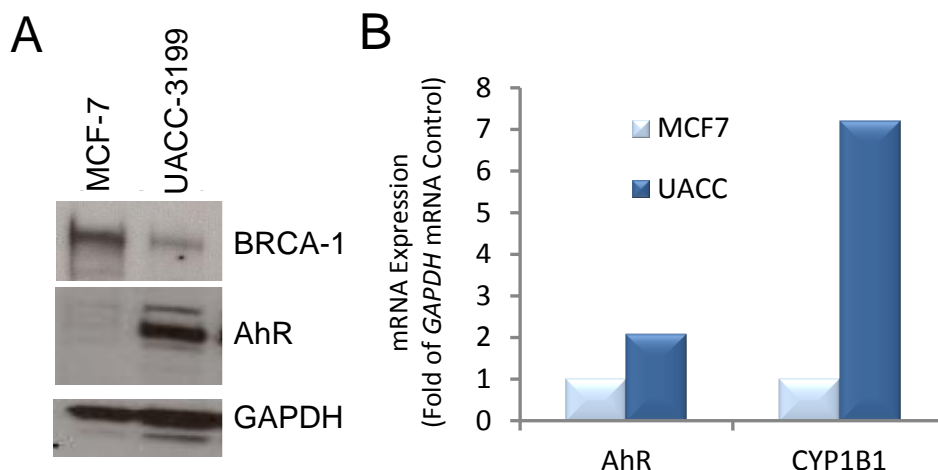


Figure 3. Reduced expression of BRCA-1 is associated with constitutive over-expression of AhR. In A) bands represent immunocomplexes for BRCA-1 and AhR in MCF-7 and UACC-3199 breast cancer cells. GAPDH are control bands. In B) bars represent AhR and CYP1B1 mRNA corrected for GAPDH from MCF-7 and UACC-3199 cells.

We, therefore, hypothesized that the AhR may contribute to silencing of BRCA-1 in UACC-3199 cells. We tested this idea by treating UACC-3199 cells with the AhR antagonist alpha-naphthoflavone (α NF). In previous studies, we found α NF to antagonize silencing of BRCA-1 by AhR agonists in ER α -positive breast cancer cells (13). Interestingly, we found that the treatment with α NF increased (from 1.0 to ~2.5-fold) BRCA-1 mRNA (Figure 4A) and protein (Figure 4B) levels, and these changes were also associated with upregulation of ER α (~2.0-fold) expression. Taken together, these results suggested that inhibition of AhR activity favored re-expression of both BRCA-1 and ER α in ER α -negative sporadic breast tumor cells.

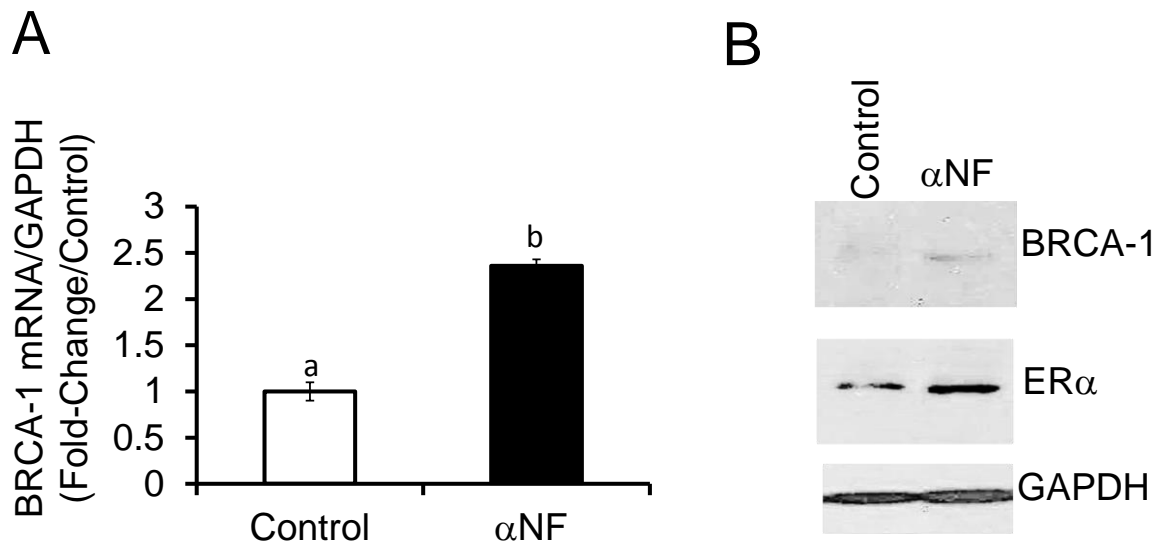


Figure 4. Antagonist of AhR reactivates BRCA-1 expression in human breast cancer cells with hypermethylated BRCA-1 promoter. In A) treatment with α NF (2 μ M) for 72 h increases BRCA-1 mRNA expression in UACC-3199 breast cancer cells. In B) α NF reactivates BRCA-1 and ER α protein expression in UACC-3199 breast cancer cells.

❖ Accomplishment 3

We acquired the mice necessary to develop the following colonies through the Experimental Mouse Shared Services (EMSS) of the University of Arizona Cancer Center. The colonies are AhR and BRCA-1 conditional knock-out models expressing Cre recombinase in the mammary gland; the groups are as follows:

1. WT BRCA-1 and expressing AhR (BRCA-1^{+/+}, AhR^{+/+});
2. WT BRCA-1 and AhR-knockout (BRCA-1^{+/+}, AhR^{-/-});

3. Heterozygous BRCA-1 expressing AhR (BRCA-1^{+/-}, AhR^{+/+});
4. Heterozygous BRCA-1 with AhR-knockout (BRCA-1^{+/-}, AhR^{-/-}).

Work is in progress to generate sufficient animals for each genotype and subject them to gestational exposure with an AhR agonist. Then, female offspring will be sacrificed to test the impact of genotype, and timing of exposure on mammary tumorigenesis.

Synopsis

Overall, results obtained during this reporting period support the hypothesis overexpression/activation of the AhR is uniquely related to development of breast tumors that are TBNC. To our knowledge, this is the first evidence linking increased expression of AhR to BRCA-1 promoter methylation in human TNBC. Also, we have developed the necessary tools and gathered information that BRCA-1 promoter methylation is likely involved in the repression of BRCA-1 expression in human tumors; and finally, we learned that targeting of the AhR with antagonists may provide a new strategy for reactivation of BRCA-1 and ER α expression. Moving forward, we will further test these inferences in animal tissues with different BRCA-1 and AhR genotype and results are expected to be available for the next report.

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- **What opportunities for training and professional development has the project provided?**

This project provided a training opportunity for Mr. Adam Lyon, who earned a Master of Science from the University of Arizona. Mr. Lyon assisted with qRT-PCR analyses. Through these activities he acquired laboratory skills to perform expression analyses. Also, we advanced our laboratory techniques for the determination of BRCA-1 promoter methylation in mammary tumors. These techniques will be crucial going forward to testing changes in BRCA-1 promoter methylation.

Additional opportunities for development included:

- Attendance by Dr. Romagnolo to the Arizona Cancer Center Cancer Biology Program Retreat, October 4, 2014;
- Attendance by Dr. Romagnolo to the Breast Cancer Workshop organized on January 24, 2015 by The Arizona Cancer Center in Tucson, AZ;

- Attendance to Arizona Cancer Center Retreat by Dr. Romagnolo and Dr. Selmin on April 10, 2015.
- **How were the results disseminated to communities of interest?**
 - Presentation of a seminar by Dr. Romagnolo to the Department of Nutritional Sciences Seminar Series, The University of Arizona, October 6, 2015.
 - Presentation by Dr. Romagnolo of instructional lectures on BRCA-1 and promoter regulation, Department of Nutritional Sciences.
 - Description of research was included in the Arizona of Cancer Center Newsletter, a periodical sent to the friends and supporters of the Arizona Cancer Center.
- **What do you plan to do during the next reporting period to accomplish the goals?**

Mammary tissues from female mice groups with various genetic backgrounds will be harvested and analyzed for BRCA-1/ER α expression methylation. Also we will perform analyzes of various genes associated with TNBC and AhR.

4. **IMPACT**

- **What was the impact on the development of the principal discipline(s) of the project?**

This project has broad implications for understanding what makes the breast susceptible to cancer. Compounds that activate the AhR include environmental xenobiotics, dietary agents, metabolites of fatty acids, and photoproducts generated in the skin from ultraviolet radiation. Data generated by this project will clarify the impact of specific activation of the AhR and interaction with BRCA-1 genotype on establishment of CpG methylation signatures associated with the development of TNBC, for which prospects for prevention and treatment remain unclear. A possible impact may be the development of specific TNBC prevention/treatment strategies based on antagonists of the AhR

What was the impact on other disciplines?

- The findings reported so far are likely to make an impact on other disciplines such as those that 1) focus on targeted AhR drug design; 3) nutritional sciences designed for breast cancer prevention based on foods

that possess anti-AhR activities; 3) development of diagnostic tools for TNBC.

- **What was the impact on technology transfer?** Nothing to report.
- **What was the impact on society beyond science and technology?** Impact beyond the bounds of science, engineering, and the academic world have been:
 - Reaching out to general public and breast cancer interest groups and patients who have expressed interest in the research as well as desire to learn more about the potential applications of our research findings.
 - Based on the knowledge that AhR-activating compounds are present in foods; are generated through uv light exposure of skin; and metabolism of certain dietary fatty acids, the findings presented here have the potential to affect behavior related to sun exposure and food practices as well as increase awareness about the risk of exposure to environmental xenobiotics that activate the AhR (i.e. polycyclic aromatic hydrocarbons, dioxins, etc).

5. **CHANGES/PROBLEMS:** *Nothing to Report.*

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

- **Journal publications.**

Romagnolo AP, Romagnolo DF, Selmin OI. BRCA1 as target for breast cancer prevention and therapy. *Anticancer Agents Med Chem.* 2015;15(1):4-14.

Selmin OI, Papoutsis JA, and Romagnolo DF. *BRCA-1* promoter CpG hypermethylation as biomarker of mammary tumorigenesis associated with increased expression and activation of AhR. *BMC Cancer*, 2015 (submitted August 12, 2015).

- **Books or other non-periodical, one-time publications.**

Romagnolo DF, and Selmin OI. Co-Editors. *Mediterranean Diet: Impact on Health and Disease.* Humana Press/Springer (2015, In Press).

Romagnolo DF and Selmin OI. Opportunities and Challenges for Adoption of the Mediterranean Diet. In: *Mediterranean Diet: Impact on Health and Disease.* 2015, Humana Press/Springer Pubs (In press).

Romagnolo DF and Selmin OI. Epigenetics of Endocrine Tumors in Women and Dietary Prevention. In: Preventive Nutrition, Fifth Edition, Eds. Adrienne Bendich, Ph.D. (Editor), Richard J Deckelbaum, M.D. (Editor), Humana Press/Springer (In press).

- **Other publications, conference papers, and presentations.** Nothing to report.
- **Website(s) or other Internet site(s)**
<http://uacc.arizona.edu/news/a-perfect-union>
 This is a web link to the University of Arizona Cancer Center. It highlights the research supported by this project and makes specific mention of the support received by the Expansion Award.
- **Technologies or techniques.** Nothing to report.
- **Inventions, patent applications, and/or licenses.** Nothing to report.

Other Products. Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Donato F Romagnolo, PhD University of Arizona	PI.
Ornella I. Selmin, PhD University of Arizona	Co-PI
Adam Lyon University of Arizona	Graduate Student. Assisted with RNA extractions and performance of qRT-PCR.
Micah Donovan	Graduate Student. Assisted with Western blotting analysis, RNA extractions, BRCA-1 promoter methylation from tumor samples.

Andreas Papoutsis, PhD	Postdoc. Assisted with preparation and submission of BRCA-1 manuscript
Christina Laukitis, MD The University of Arizona	Assisted with design of experiments with human breast tumors; organization of tumors based on histo-pathological characteristics;
Tom Doetschman, PhD The University of Arizona	Collaborator on design of experiments with mouse models.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Nothing to report.
- **What other organizations were involved as partners?** Nothing to report, all personnel was from The university of Arizona.

7. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Nothing to report.
- **QUAD CHARTS:** Nothing to report.

8. APPENDICES: Nothing to report.